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(54) **Process for the production of vanillin**

(57) The present invention concerns a microbiological process for the production of vanillin, comprising cultivating in a nutrient broth of a bacterium belonging to the order of the Actinomycetales, preferably of the family Streptomycetaceae, adding the substrate ferulic acid, wherein the substrate concentration in the nutrient broth is from about 5 gL^{-1} to about 40 gL^{-1} in the fermentation broth, producing vanillin as the main reaction product of the biotransformation of ferulic acid and separating the biomass from the fermentation broth, and extracting the vanillin and, if desired, the by-product guaiacol from the fermentation broth.

Description

The present invention concerns a microbiological process for the production of vanillin from ferulic acid. According to this process, a culture, preferably a submerged culture, of any bacterium of the order Actinomycetales, preferably of the family Streptomycetaceae is incubated with the substrate ferulic acid to fermentatively produce vanillin. The product vanillin is recovered from the fermentation broth by a designed extraction method allowing also the separation and recovery of valuable fermentation by-products, in order to obtain the analytically and sensorically purified product vanillin, and, in particular the by-product guaiacol.

In the use of flavouring compounds, it is more and more important that the flavour compounds can be designated as "natural". In line with the European and U.S. regulations this means that the compound has to be obtained by physical, enzymatic or microbiological processes and only from materials of plant or animal origin. Various research activities during the last decade were thus focused on the use of renewable, cheap and natural raw material sources for the fermentative production of vanillin. However, in publications and in patents, commercially attractive volumetric yields were very rarely reported so far.

Guaiacol is a phenolic, smoky type of molecule which significantly contributes to the characteristic flavour of vanilla extracts. It is thus often used in combination with vanillin for vanilla type flavours. However, the fermentative production of natural guaiacol has not yet been described so far.

In the past 10 years, several patent applications have been filed concerning the microbial or enzymatic production of vanillin. In general, a suitable precursor is transformed to vanillin by a microorganism or an enzyme. Suggested precursors are eugenol, isoeugenol, ferulic acid, curcumin or benzoe siam resins. Usually, transformation yields are extremely low. Examples are e.g. Haarman & Reimer (EP 0 405 197 A1) claiming a production of 18 mgL^{-1} starting from 0.2 gL^{-1} eugenol using the microorganisms Serratia, Klebsiella or Enterobacter. This transformation furthermore takes 13 days. Pernod-Ricard (EP 453 368 A) claim 46 mgL^{-1} vanillin obtained in a 6 days Pycnoporus fermentation from ferulic acid. In this line is also Kraft General Foods (US 5,128,253) claiming 210 mgL^{-1} vanillin from ferulic acid within 54 days. In order to obtain this titer a reducing agent had to be added as otherwise the formation of vanillin would not occur and only vanillic acid would be formed. Takasago (JP 227980/1993) prepared mutants of *Pseudomonas* strains that are blocked in the degradation pathway of vanillin. Thus, starting with 1 gL^{-1} ferulic acid 0.28 gL^{-1} vanillin could be obtained. The so far sole application reporting economically attractive volumetric yields of vanillin in a fermentation process has recently been published by Haarman & Reimer (EP 0 761 817 A2). They identified two strains of the genus Amycolatopsis which are able to accumulate vanillin up to a concentration of 11.5 gL^{-1} in the fermentation broth after feed of ferulic acid.

In conclusion, it can be stated that high amounts of vanillin are not easily formed in microbial systems. This is mainly due to the cellular toxicity of vanillin, which at concentrations above 1 gL^{-1} prevents growth of the vanillin producing microorganisms. In microbial systems, usually the respective alcohol or acid is found and not vanillin. This toxic effect of vanillin was overcome by the use of enzymes (Quest, EP 0 542 348 A2). Treating isoeugenol with lipoxygenase resulted in 10 - 15 gL^{-1} vanillin at a yield of 10 - 15%. Much lower concentrations were obtained when using eugenol (0.3 - 0.5 gL^{-1} in a yield of 0.3 - 0.5%) and no turnover is reported for ferulic acid. The method employing lipoxygenase is scarcely attractive from the economic point of view.

Another measure to circumvent the toxicity of this compound is the microbial production of coniferylaldehyde which forms vanillin upon thermal treatment, see BASF (Offenlegungsschrift, DE 3604874 A1). Similar is also the immobilized cell system as described in the recent Orsan patent application (WO 96/34971) in which vanillin is accumulated up to a concentration of 1 gL^{-1}. A possible economic benefit of using immobilized biomass is given by recycling the biocatalyst.

Many papers deal with the respective metabolic pathways starting from eugenol, isoeugenol or ferulic acid. In general, vanillin is believed to be an intermediate compound in the degradation pathway of these compounds. Two publications may be cited showing the involvement of vanillin in the degradation of ferulic acid. Toms and Wood, Biochemistry 9 (1970) 337-43, cultivated *Pseudomonas sp.* on ferulic acid and elucidated the degradation pathway. Though vanillin was not found in the culture supernatant, evidence was given that vanillin is an intermediate compound, since vanillic acid could be detected. Starting from ferulic acid, vanillin was obtained in cultures of Streptomyces setonii (Sutherland et al., Can. J. Microbiol. 29 (1983) 1253-57). No indication was given on the amount, but only traces where found when repeating the experiment.

Ferulic acid as a substrate for biotransformations is abundantly available from different natural sources. The acid often occurs in the form of a glucoside in plant materials, such as wood, sugar beet melasse, bran of corn, rice and various types of grasses. It can be isolated from the corresponding glycosides in these products by well-known hydrolysis methods, e.g. using enzymes, and can be used as crude material or purified material. A British source (GB 2301103 A1) describes for instance the enzymatic breakdown of ferulic acid containing plant material by a ferulic acid esterase, in order to obtain the free acid.

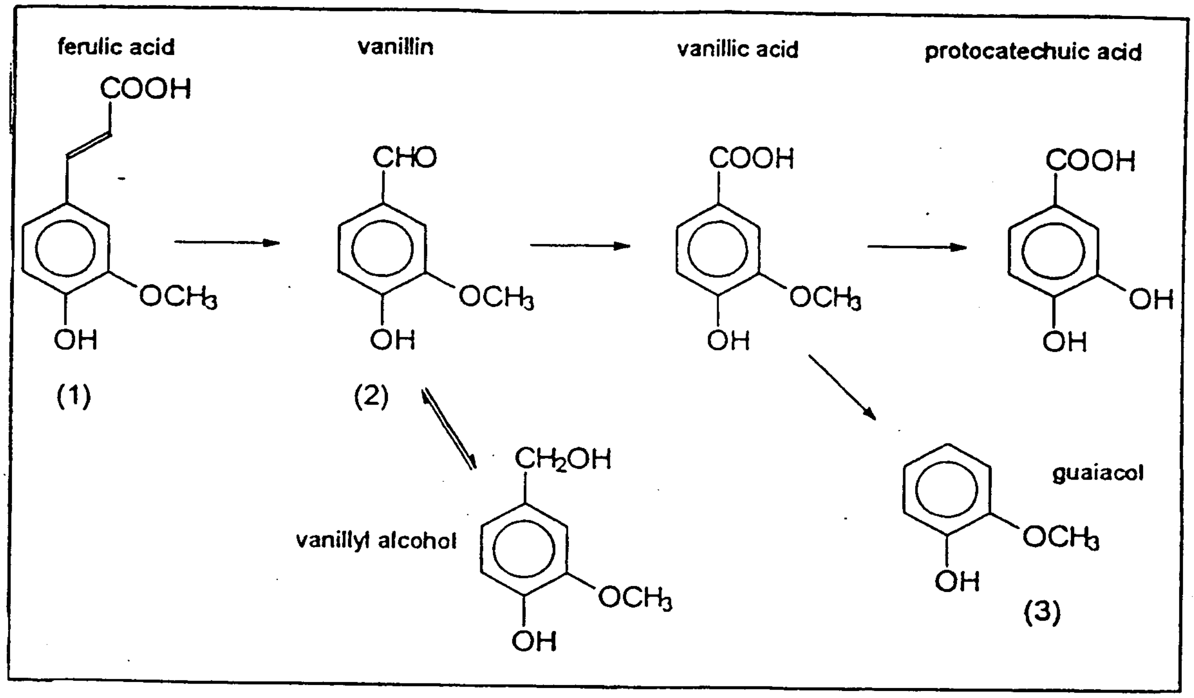
The present new, high-yield microbiological process for the production of vanillin comprises cultivating first in a nutrient broth a microorganism of the order of the Actinomycetales, preferably of the family Streptomycetaceae, most

preferably the bacterium Streptomyces setonii, wherein, preferably, the cultivating period is about 5-40 hours and lasts, until the carbon source glucose is (almost) consumed, then adding the substrate ferulic acid in the range of about 5-40 gL^{-1} of fermentation broth, either continuously or batch-wise. After an approximate incubation (biotransformation) period of about 5-50 hours, substrate conversion to vanillin and several by-products is completed. The ferulic acid is consumed and vanillin accumulated up to ca. 8-16 gL^{-1} in the fermentation broth. Typical by-products of the ferulic acid biotransformation are vanillic alcohol, vanillic acid, guaiacol, *para*vinylguaiacol and 2-methoxy-4-ethyl-phenol.

Subsequent product recovery consists in the removal of the biomass, conveniently followed by a two-step extraction with an appropriate organic solvent, preferably methyl-tert-butylether. A first extraction is carried out at a pH > ca. 9, preferably at a pH of 10 to ca. 11 in the aqueous phase to selectively extract by-products, such as the sensorically highly active guaiacol. Then, the aqueous raffinate is "acidified" to neutral pH values to selectively extract the product vanillin. Purification of the raw vanillin extract may finally be done by applying well-known recrystallization methods. Guaiacol may be purified from the raw extract by distillation.

The microbiological process and the extraction procedure described are useful for the economically attractive production of natural vanillin as well as by-products from ferulic acid according to the following biochemical pathway:

Pathway of ferulic acid degradation, e.g. by Streptomyces setonii



The resulting vanillin (compound 2) as well as the by-product guaiacol (compound 3) are both known flavour and fragrance compounds. Their use and application are known to the man skilled in the art. By using effective and balanced amounts of these compounds it is possible to augment or enhance the organoleptic properties of flavoured consumables, such as beverages, dairy products, baked goods, ice cream and the like. Fermentatively produced vanillin and guaiacol are especially valuable in any vanilla-type and fruit flavour compositions where entirely natural ingredients are required.

As pointed out above, exact fermentation conditions combined with an effective product recovery method have now been discovered to allow a high yield production of sensorically and analytically purified vanillin as well as getting the high-impact flavour compound guaiacol as a by-product. These conditions are based upon the cultivation of the bacterium of the genus Streptomyces in an appropriate culture medium and the subsequent addition of the substrate ferulic acid at excess concentrations, i.e. ca. 5 to ca. 40 g/l, to obtain vanillin at high volumetric yields in the fermentation broth.

Most suitable is, as pointed out above, the species Streptomyces setonii, preferably the commercially available strain ATCC 39116.

The substrate ferulic acid is defined by formula (1). According to the novel process a ferulic acid material with a ferulic acid content of, preferably, more than 10 % is used as substrate. The nature of the remaining compounds is depending on the source.

In carrying out the present invention, cultivation of the bacterium is carried out in an aqueous medium in the presence of usual nutrient substances. A suitable culture medium contains a carbon source, an organic or inorganic nitrogen source, inorganic salts and growth factors.

For the culture medium, glucose is preferably used as the carbon source, e.g. at a concentration of ca. 5 - 50 gL^{-1}, preferably ca. 20-35 gL^{-1}. Yeast extract is a useful source of nitrogen, phosphates, growth factors and trace elements may be added, e.g. at preferred concentration of ca. 2-20 gL^{-1}, most preferably ca. 5-10 gL^{-1}. In addition, magnesium ions, e.g. magnesium sulfate may be added at a concentration of ca. 0.1-5 gL^{-1}, preferably at ca. 0.5-1 gL^{-1}.

The culture broth is prepared and sterilized in a bioreactor, and is then inoculated with a Streptomyces strain in order to initiate the growth phase. An appropriate duration of the growth phase is about 5-40 hours, preferably about 15-35 hours and most preferably about 20-30 hours.

Specifications of further process conditions are:

pH-range:	ca. 7 to ca. 9
temperature range:	ca. 30 to ca. 45°C
aeration:	is preferred for this aerobic process
stirring:	is preferred.

After the termination of the growth phase, the substrate ferulic acid is fed to the culture. A suitable amount of substrate-feed is 5-40 gL^{-1} of fermentation broth, preferably about 15-30 gL^{-1}, most preferably 20-25 gL^{-1}. The substrate is either fed as solid material or as aqueous solution or suspension. The total amount of substrate is either fed in one step, in two or more feeding-steps, or continuously.

The biotransformation phase starts with the beginning of the substrate feed and lasts about 5-50 hours, preferably 10-30 hours and most preferably 15-25 hours, namely until all substrate is converted to product and by-products.

It is assumed that an excess concentration of the fed ferulic acid is mainly responsible for the high volumetric yield of vanillin as observed after the terminated substrate conversion. In addition, the process conditions outlined above are also assumed to be responsible for the accumulation of the valuable material guaiacol.

After the terminated biotransformation phase, the biomass is separated from the fermentation broth by any well known method, such as centrifugation or membrane filtration and the like to obtain a cell-free fermentation broth.

Since the biotransformation converts the hydrophilic substrate ferulic acid into rather hydrophobic substances such as vanillin and guaiacol, the overall volumetric productivity of the fermentation system might be increased by applying any in-situ product recovery method. For this purpose, e.g. an extractive phase can be added to the fermentation broth using, e.g. a water - immiscible - organic solvent, a plant oil or any solid extractant, e.g. a resin preferably, neutral resin such as Amberlite XAD 4 or XAD 7 or the like. Such an in-situ product recovery method might allow continuing formation of vanillin and guaiacol also after having reached the water solubility concentrations.

From this fermentation broth, vanillin and the by-products may now be extracted selectively by two different extraction methods:

Continuous liquid-liquid extraction a) or batch wise extraction b) are suitable.

a) On the basis of an extraction dependent on the pH value, an efficient isolation of vanillin and also of guaiacol can be carried out. In a first step, guaiacol can be extracted from an aqueous fermentation broth. For this step, a counter-current extraction method is preferably used, preferably in a extractor, by means of an organic water insoluble solvent. Examples of solvents are esters of C_{1-3} acids with C_{1-4}-alcohols, ethers, in particular methyl tert-butyl ether (MTBE). The pH is preferably between 10 to 11, in particular pH 10,8 - 11.
Vanillin is thereafter extracted from the aqueous raffinate of the guaiacol extraction at pH values of ca. 5 to ca. 8, preferably ca. 6 to ca. 7,5, most preferably between ca. 6,9 to ca. 7,1.
Working with vanillin concentrations of ca. 8 to ca. 16g/l, the counter current extraction operates most suitably in a ratio of feed/solvent of ca. 2,5 - 3 : 1, in particular ca. 2,6 : 1.

b) At higher concentrations of vanillin in the aqueous phase, e.g. after a concentration of the fermentation broth by means of water evaporation, a corresponding two-step batch-wise extraction at different pH values and with the solvents proposed above is suitable, and preferable.

The advantages of the novel process can be summarized as follows:

(1) Fermentation conditions are available which enable the accumulation of vanillin in the fermentation broth of

Streptomyces, e.g. S. setonii to economically attractive concentrations (ca. 8-16 gL^{-1}).

(2) The process enables the simultaneous production of vanillin and guaiacol, i.e. two products of high value in the natural flavour preparation.

(3) The fermentation process is of low technical complexity and uses raw materials from easily accessible sources.

Finally, the invention concerns also the novel process for the manufacture of vanillin, but using instead of Streptomyces setonii ATCC 39116, its enzymes or any recombinant microorganisms, e.g. yeast, which contain the genetic material coding for the enzymes which are relevant or involved in the cellular biosynthesis of vanillin and/or guaiacol - and thus not the microoroganism as such.

Example 1

250 mL shake flasks containing 50 mL of the following medium were prepared: 103 gL^{-1} sucrose, 4 gL^{-1} Na_2HPO_4, 1 gL^{-1} KH_2PO_4, 1 gL^{-1} yeast extract, 0.2 gL^{-1} NaCl, 0.2 gL^{-1} $MgSO_4$ and 0.05 gL^{-1} $CaCl_2$. The pH was adjusted to 7.2 using NaOH. A shake flask was inoculated with 2 mL of preculture of Streptomyces setonii ATCC 39116 and cultivated at 37°C, 190 rpm for 16 hours. At the end of the growth phase 0.3 g ferulic acid (purchased from Aldrich, cat. no. 12.870-8, 99%) was added to the culture. For this purpose a 10 % w/w solution of the acid substrate in 0.5 M NaOH (final pH of the solution was approximately 7.2) was previously prepared and sterile-filtered. The flask was incubated again at 37°C, 190 rpm. After 31.5 hours of biotransformation (incubation) a vanillin concentration of 3.10 gL^{-1} (HPLC) was reached. A molecular yield of 66 mol % was calculated.

Example 2

A 250 mL shake flask was prepared and incubated as in Example 1. After a 16 hours growth phase 0.6 g ferulic acid (as a 10 % w/w solution in 0.5 M NaOH) was added to the culture. The flask was incubated again at 37°C and 190 rpm. After 78 hours of biotransformation (incubation) a vanillin concentration of 5.94 gL^{-1} (HPLC) was reached, corresponding to a yield of 63 mol %.

Example 3

A 250 mL shake flask was prepared and incubated as in Example 1. After a 18 hours growth phase 0.3 g ferulic acid (as a 10 % w/w solution in 0.5 M NaOH) was added to the culture. The flask was incubated again at 37°C and 190 rpm. After 28 hours a second feed of 0.3 g ferulic acid followed. At the end of the incubation (58 hours) a vanillin concentration of 6.41 gL^{-1} was reached, corresponding to a yield of 68 mol %.

Example 4

A preculture of Streptomyces setonii was grown in a shake flask at pH 7.2, 37°C, 190 rpm, for 24 hours. The shake flask medium contained 5 gL^{-1} glucose, 4 gL^{-1} Na_2HPO_4, 1 gL^{-1} KH_2PO_4, 10 gL^{-1} yeast extract, and 0.2 gL^{-1} $MgSO_4$.

A bioreactor was filled with 10 L of a medium containing 32 gL^{-1} glucose, 8 gL^{-1} yeast extract, 0.8 gL^{-1} $MgSO_4$ and 0.2 gL^{-1} antifoam agent (Dow Corning AF 1520). After thermal sterilization the reactor was inoculated with the previously grown shake flask preculture. The amount of inoculum used was 3%. The process conditions were 37°C, pH 7.2, airflow rate 1.0 vvm, 800 rpm. After 24 hours of growth phase a remaining glucose concentration of 4.6 gL^{-1} was measured. Subsequently, the pH was shifted to 8.5 using NaOH (30%) and 24.5 hours after inoculation 2.25 L of a 10% w/w solution of ferulic acid in 0.5 M NaOH was added to the fermentation broth. At the time of the feed, the glucose concentration was down to 4.0 gL^{-1}. 3-4 hours after the addition of the precursor the beginning of the biotransformation of ferulic acid to vanillin was observed. 17 hours after the precursor feed, concentrations of 13.9 gL^{-1} vanillin and 0.4 gL^{-1} guaiacol were measured in the fermentation broth by GC. At that time, the ferulic acid was completely converted. A yield of vanillin of 75 mol % was calculated.

The bioprocess was then terminated by pasteurization at 80°C for 15 minutes. The fermentation broth was microfiltered (0.2 μm).

Example 5

A 450 L bioreactor with a working volume of 340 L was run according to the procedure described in the previous example.

After a growth period of 26.5 hours the pH was shifted to 8.5 and a first ferulic acid feed amounting to 4.08 kg was carried out according to Example 4. At that time a remaining concentration of 7.5 gL^{-1} glucose was measured. 1 hour later a another 3.57 kg of precursor was fed. The total amount of ferulic acid addition was 22.5 gL^{-1}. 25.5 hours after the first precursor feed a vanillin concentration of 9.0 gL^{-1} was measured. The ferulic acid concentration was now 1.75 gL^{-1}. A yield of vanillin of 51 mol % was obtained.

Example 6

Liquid-liquid countercurrent extraction of vanillin and guaiacol at technical scale.

7930 kg of cell-free, membrane-filtered fermentation broth containing 7.1 gL^{-1} vanillin and 0.35 gL^{-1} guaiacol were adjusted to pH 11 with NaOH and extracted first with MTBE as solvent in a stirred-chamber counter current extractor to separate the guaiacol. After MTBE evaporation, 8 kg of raw extract containing MTBE and 33 % w/w guaiacol were recovered. The pH of the aqueous raffinate of this alkaline extraction was then shifted to 6.9 - 7.1 with hydrochloric acid and again extracted with MTBE in the same extractor to separate the vanillin. From this second extraction step, 150 kg of a raw extract containing MTBE and 37% w/w vanillin were obtained.

The figure represents:

A typical chart of a vanillin production batch at the 10 L scale.

Δ vanillin concentration [gL^{-1}]; -▽- ferulic acid concentration [gL^{-1}]; X guaiacol concentration [gL^{-1}]; -o- pH value; -❑- pO_2 value [%]; -x- glucose concentration [gL^{-1}]. After a 24 hours growth phase the pH was adjusted to 8.5 before the ferulic acid was fed. 3-4 hours upon the substrate addition a small amount of vanillin was detected. A vanillin concentration of 13.9 gL^{-1} was reached after totally 41 hours of fermentation (17 hours after the ferulic acid feed). At that time a guaiacol concentration of 0.38 gL^{-1} was measured. A production rate of 1.10 $gL^{-1}h^{-1}$ for vanillin and of 0.04 $gL^{-1}h^{-1}$ for guaiacol were calculated respectively. After complete conversion of ferulic acid a decrease in the concentrations of vanillin and guaiacol was observed. Quantitative measurements were carried out by HPLC and GC.

Claims

1. A process for the manufacture of vanillin, comprising

   a) cultivating in a nutrient broth of a bacterium belonging to the order of the Actinomycetales, preferably of the family Streptomycetaceae, most preferably Streptomyces setonii, then
   b) adding the substrate ferulic acid, wherein the substrate concentration in the nutrient broth is from about 5 gL^{-1} to about 40 gL^{-1} in the fermentation broth, producing vanillin as the main reaction product of the biotransformation of ferulic acid and separating the biomass from the fermentation broth,
   c) and, extracting the vanillin to produced and, if desired, the by-product guaiacol from the fermentation broth.

2. A process according to claim 1, wherein, in step c) the guaiacol is first extracted by increasing the pH of the fermentation broth to ca. >9 and by using an organic solvent.

3. A process according to claim 2, wherein the pH of the fermentation broth is subsequently brought to a value of ca. 7 and the vanillin is extracted by means of an organic solvent.

4. A process according to claim 1, wherein, the reaction products vanillin and guaiacol are extracted under neutral conditions.

5. A process according to claim 1, wherein the cultivation is carried out for ca. 5 to ca. 40 hours.

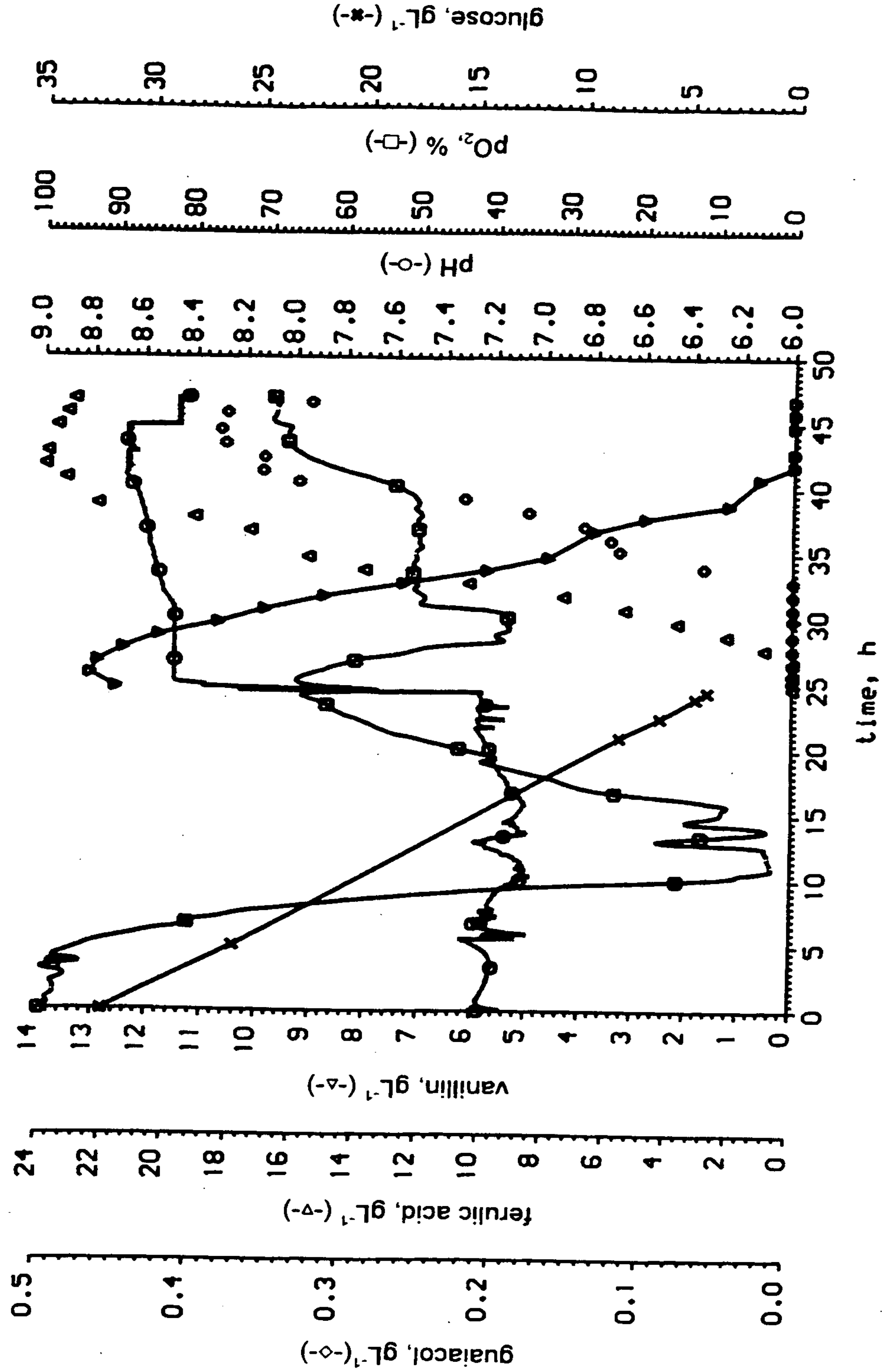
6. A process according to any one of the preceding claims, wherein methyl-tert.butyl ether is the extraction solvent.

7. A process according to any one of claims 1 to 6, wherein the bio-transformation period is ca. 5 to ca. 50 hours.

8. A process according to any one of the preceding claims, using, instead of Streptomyces setonii, e.g. Streptomyces setonii ATCC 39116, its enzymes or any recombinant microorganisms e.g. yeast which contain the relevant genetic material coding for the enzymes, which are relevant or involved in the cellular biosynthesis of vanillin and/or guaiacol.

Figure

production of vanillin and guaiacol



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EUROPEAN SEARCH REPORT

Application Number
EP 98 11 0765

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X	WO 96 08576 A (INSTITUT NATIONAL DE LA RECHERCHE AGRONOMIQUE - I.N.R.A.) 21 March 1996 * the whole document * ---	1-8	C12P7/24 C12P7/22 //(C12P7/24, C12R1:465), (C12P7/24, C12R1:01), (C12P7/22, C12R1:465), (C12P7/22, C12R1:01)
X,D	J. B. SUTHERLAND ET AL.: "Metabolism of cinnamic, p-coumaric, and ferulic acids by Streptomyces setonii." CAN. J. MICROBIOL., vol. 29, 1983, pages 1253-1257, XP000650324 * the whole document * ---	1-8	
X,D	WO 96 34971 A (ORSAN) 7 November 1996 * the whole document * ---	1-8	
X,D	EP 0 761 817 A (HAARMANN & REIMER GMBH) 12 March 1997 * the whole document * ---	1-8	
A,D	US 5 128 253 A (KRAFT GENERAL FOODS, INC.) 7 July 1992 * the whole document * -----	1-8	**TECHNICAL FIELDS SEARCHED (Int.Cl.6)** C12P

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
MUNICH	21 September 1998	Douschan, K

CATEGORY OF CITED DOCUMENTS

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